

Application No. 09/627,787  
Attorney Docket No. 02481.1679

B<sup>2</sup>

b) incubating the conjugate with the membrane, where upon

c) the conjugate is transported across the membrane.

### REMARKS

#### **Status of the Claims**

Claims 1-26 are pending in this application. The Office has withdrawn claims 6 and 7 as being drawn to non-elected subject matter. Applicants acknowledge the Office's statement that, upon allowance of a generic claim, Applicants will be entitled to consideration of claims to additional species written in dependent form or that otherwise include all of the limitations of an allowed generic claim. (Office Action, page 2.)

Applicants have amended claim 6 to delete the phrase "is a low molecular weight compound having." As amended, claim 6 encompasses compounds with a molecular weight less than 500 Daltons. Because the specification indicates that low molecular weight compounds, for example, have a molecular weight less than 500 Daltons, this amendment does not alter the scope of claim 6. (Page 8, lines 19-21.) Applicants have also amended claim 16 to add step c) in which "the conjugate is transported across the membrane." This amendment is supported by the specification as a whole. Support for the amendment may also be found, for example, at page 23, line 5, to page 24, line 27; page 31, Example 7; pages 36-37, Examples 16-18; and pages 37-41, Tables 1-6. Because these amendments do not introduce new matter or require a further search of the art, Applicants respectfully request their entry.

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### **Claims 22-24 Are Enabled by the Specification**

The Office contends that claims 22-24, directed to a "process for preparing a pharmaceutical composition" and to a "pharmaceutical composition" are not enabled. (Office Action, pages 3-9.) The Office contends that antisense is an unpredictable technology and asserts that, because the claimed conjugates may comprise antisense oligonucleotides, using the claimed compositions requires undue experimentation.

Applicants traverse this rejection. Claim 22 part a) requires attaching the conjugate to a "pharmaceutically active compound or a derivative thereof." That is, by the plain language of claim 22, the antisense component of the composition is "pharmaceutically active." The level of predictability of antisense technology in general is therefore irrelevant to determining whether claims 22-24 are enabled by the specification.

The working examples of the specification show that the present invention improves the cellular uptake of several different oligonucleotides. (Specification, Examples 7 and 16-18, and Tables 1-6.) For instance, Example 7 and Tables 1-3 show that fluorescence from FDA-labeled CO\_1 and CO\_3 oligonucleotides, which are conjugates according to the invention, is moderate to strong after 120 minutes of incubation with mammalian, insect, fungal, and prokaryotic cells. In contrast, these cells do not generally take up fluorescein-labeled oligonucleotides of the same sequence that have not been prepared as conjugates according to the invention. (*Id.*) Example 16 and Table 5 show that mammalian REH cells effectively take up 20, 50, and 80-mer oligonucleotides that have been modified according to the invention. Example 17, Figure 9, and Table 4 show that an oligonucleotide conjugate inhibited tumor cell growth in culture.

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The Office has provided no evidence to support its assertion that one skilled in the art would have to engage in undue experimentation in order to use the claimed conjugates to increase the cellular uptake of other "pharmaceutically active" oligonucleotides known in the art. For example, some of these pharmaceutically active oligonucleotides are described in a recent review by Alan Dove. (*Nature Biotechnology* 20: 121-124 (2002); Exhibit A.) Table 1 in that review includes a list of compounds presently in phase II-III clinical trials. Although the Dove review was published after Applicants' July 28, 1999, priority date, it is reasonable to conclude that some, if not all, of these oligonucleotides were known to be "pharmaceutically active" at the time the present application was filed because clinical testing frequently takes several years. These molecules target a variety of conditions, including various cancers, viral infections, diabetes, and asthma. (Exhibit A, Table 1.) The review also discusses Vitravene, a compound approved by the Food and Drug Administration in August 1998 for treatment of cytomegalovirus. (Exhibit A, page 122.) Moreover, Table 1 of the review by Crooke and Lebleu shows that a large number of genes have been successfully targeted with antisense oligonucleotides. Commenting on this table, Crooke and Lebleu state at page 24 that "[t]his array of pharmacological effects clearly demonstrates the broad potential therapeutic applicability of these drugs." The specification at page 13, line 21, to page 17, line 26, also lists numerous sequences that are directed against different gene targets. The Office has provided no reason to suppose that it would require undue experimentation to make and use the claimed conjugates with such pharmaceutically active oligonucleotides.

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Applicants submit that the conjugation method of the invention can be generally applied to oligonucleotides and several other types of molecules. (Specification, page 5, lines 21-34; page 22, lines 1-12; and Examples 2-4.) Testing whether the cellular uptake of the molecules is improved by conjugation according to the invention involves relatively simple fluorescence assays using cell culture systems well known to those of skill in the art. For example, the review by Crooke and Lebleu, which the Office has cited, describes tests of cellular uptake of antisense oligonucleotides in HeLa cells and *Trypanosome brucei*. (S.T. Crooke & B. Lebleu, *Antisense Research and Applications*, (CRC Press, 1993), pages 19-21 and Table 1.) Thus, the working examples establish a sufficient correlation with the expected use of the claimed pharmaceutical compositions. See M.P.E.P. §§ 2107.03(III) and 2164.02. Using the claimed conjugates to improve the cellular uptake of pharmaceutically active compounds is therefore credible and involves no undue experimentation.

The Office also asserts that the specification lacks disclosure of particular therapeutic protocols, dosages, or routes of delivery for therapeutic antisense oligonucleotides. (Office Action, pages 6-7.)

Applicants submit that it is not necessary to provide guidance in the specification as to particular therapeutic protocols because the invention provides a method for increasing the cellular uptake of "pharmaceutically active" antisense components for which this information is already known. Where an invention is an improvement of a known method, as here, there is no need to re-state the known details of the method in the specification. Indeed, the specification need not disclose, and preferably omits, what is well known to those skilled in the art and already available to the public.

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M.P.E.P. § 2164.05(a). For example, therapeutic protocols, dosages, and routes of delivery must be known by those of skill in the art for the antisense oligonucleotides listed in Table I of the Dove review because these parameters are requirements for performing a clinical trial.

The Office also asserts that the claimed compositions are not enabled for use as *in vivo* diagnostics. (Office Action, pages 8-9.) The Office has cited two publications relating to tumor cell targeting in mice with peptide-nucleic acid compounds. However, those in the art have successfully used other methods of *in vivo* diagnosis with antisense oligonucleotides, such as *in situ* hybridization. For example, Y. Ishidou et al. applied a synthetic oligodeoxynucleotide probe to the skin of human patients to detect increased expression of decorin mRNA in the epidermis, an indication of a disease causing ossification of the ligaments. (*In Vivo* 9: 469 Abstract (1995); Exhibit B.) *In situ* hybridization has also been widely used in *ex vivo* procedures such as tissue biopsies. D. Gelmetti et al. used dioxigenin-labeled antisense RNA probes to detect rabbit haemorrhagic disease virus based on analysis of liver and spleen tissue. (*J. Virol. Methods* 72: 219 Abstract (1998); Exhibit C.) I. Eberhagen et al. used antisense probes to determine the level of expression of a DNA repair enzyme in human mammary carcinomas and brain tumors. (*Anticancer Res.* 15: 761 Abstract; Exhibit D.) R.P. Stowe et al. tested the peripheral blood of patients with chronic mononucleosis for the presence of the Epstein-Barr virus using fluorescein-labeled antisense oligonucleotides and flow cytometry. (*J. Virol. Methods* 75: 83 Abstract (1998); Exhibit E.) In all of the above protocols, which were published prior to Applicants' filing date, it is necessary for the antisense probe to enter the cells of the tissue or organ in order to bind and detect

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the appropriate mRNA. Because the present invention allows for improved cellular uptake of antisense oligonucleotides and other compounds, it is expected that these *in vivo* diagnostic methods can be improved by applying the instant claimed methods.

In summary, Exhibits A-E and the discussion above show that the processes of claims 22 and 23 and the pharmaceutical compositions of claim 24 are adequately supported by the specification and the prior art because they have a credible pharmaceutical utility to which the working examples correlate. Because the Office has provided no basis for the assertion that one skilled in the art would be forced to engage in undue experimentation in order to use the claimed conjugates to increase the cellular uptake of pharmaceutically active oligonucleotides, Applicants respectfully request the withdrawal of this rejection.

#### **Claims 5-7, 9, and 16 Are Definite**

The Office first contends that the phrase "the oligonucleotide is modified" renders claim 5 indefinite. (Office Action, page 9.) According to the Office, the specification does not provide a standard for determining the meaning of this phrase and one of skill in the art would not understand what the oligonucleotide is modified relative to. Applicants traverse this rejection. The specification at page 10, lines 19-25, describes oligonucleotides and the nucleotide monomers that they may comprise. It also describes numerous modifications at page 10, line 26, to page 13, line 2. Thus, the specification illustrates the oligonucleotide that is to be modified and some of the kinds of modifications that could be used. Furthermore, there is an extensive body of art concerning modified oligonucleotides, indicating that those of skill in the art understand the metes and bounds of the term. In fact, the term "modified oligonucleotide" is so

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widely used in the art that there are currently over 4300 references in the PubMed database that contain it. For example, several of the publications submitted to the Office in conjunction with this application discuss modifications that may be introduced chemically. (See, e.g., C.F. Bennett et al., P. Breeuwer et al., R.P. Iyer et al., J. Hunziker et al., E.P. Stirchak et al., J. Summerton et al., and P.E. Nielsen et al.) In addition to modifications prepared by chemical synthesis, there are a number of modifications that can be incorporated into oligonucleotides *in vivo*, such as the 7-methyl-guanosine 5'-end cap, the formation of the unusual bases of transfer RNAs, or the methylation of genomic DNA. Therefore, Applicants submit that both the specification and the art set forth the metes and bounds of this phrase.

Second, the Office contends that claims 6 and 7 are indefinite because the phrase "low-molecular-weight" is a relative expression. The Office asserts that it is not clear which compounds of < 500 Daltons are "low-molecular-weight" compounds. (Office Action, pages 9-10.) Because Applicants have amended claim 6 to remove this phrase, this rejection is moot.

The Office next asserts that the structures F1-F11 in claim 9 employ "non-standard nomenclature," i.e., a "squiggly" line, and are therefore indefinite. (Office Action, page 10.) Applicants traverse this rejection because squiggly lines are often used to denote how a particular group is attached to a larger molecule. For example, the Abstract of T.P. Prakash et al., submitted to the Office on August 22, 2001, shows two nucleotide units of an oligonucleotide. Oxygen atoms at the 3' and 5' end are drawn with a squiggly line to denote their attachment to the rest of the oligonucleotide. Figures

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3 and 7 of the article by M. Manoharan, also submitted to the Office on August 22, 2001, include similar drawings.

Moreover, Applicants submit that even if, *arguendo*, the drawings in claim 9 are not "standard," the meaning of the squiggly line is clear. The carbon to which this line is attached is the only carbon atom in each of the structures that contains only 3 bonds to other atoms within the structure. Therefore, the only chemically reasonable interpretation of the squiggly line is that it represents the point of direct or indirect attachment to the molecule to be transported.

Finally, the Office contends that claim 16 is indefinite because it allegedly contains method steps "not concordant with the purpose set forth in the preamble," which states "[a] method for transporting a molecule across a membrane." (Office Action, page 10.) Applicants submit that this rejection is moot in light of the amendment to claim 16. The amendment adds step c) in which "the conjugate is transported across the membrane."

In light of the above arguments and amendments, Applicants request that the Office withdraw each of these rejections.

#### **The Office Has an Obligation to Examine Claims 1-5 and 8-26**

The Office finds that claims drawn to the elected species, a conjugate comprising F3 as an aryl group, oligonucleotides as the compounds to be transported, and a carboxylic acid as a reactive function, are allowable. (Office Action, page 10.)

However, the Office also objects to claims 1-5 and 8-26, asserting that they embrace species other than those that Applicants have provisionally elected. (*Id.*) Under 37 C.F.R. § 1.146, the Office has a duty to examine the non-elected subject matter of these

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claims to the extent necessary to determine the patentability of the generic claims.  
M.P.E.P. § 803.02. Applicants respectfully request that the Office carry out this examination with regard to claims 1-5 and 8-26, as well as claims 6-7.

### CONCLUSION

In view of the foregoing amendment and remarks, Applicants respectfully requests the reconsideration and reexamination of this application and the timely allowance of claims 1-26.

Please grant any extensions of time required to enter this response and charge any additional required fees to our Deposit Account No. 06-0916.

Respectfully submitted,

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Dated: April 2, 2002

By: 

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**APPENDIX TO AMENDMENT OF APRIL 2, 2002**

**Version with Changes Marked-up**

**Amendment to Claims**

6. (AMENDED) The conjugate as claimed in claim 1, wherein the molecule to be transported [is a low-molecular-weight compound having] has a molecular weight < 500 Dalton.
16. (AMENDED) A method for transporting a molecule across a membrane, which comprises
- a) preparing a conjugate in which the molecule to be transported is attached to at least one aryl radical of the formula I or II, [and]
  - b) incubating the conjugate with the membrane, whereupon
  - c) the conjugate is transported across the membrane.

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## Antisense and sensibility

It took a leap of faith for researchers to believe that antisense technology might lead to novel medicines, but that commitment could soon pay off.

Alan Dove

Though elegant in theory, antisense technology has proved rather more challenging in its execution. In its short history, antisense has been hailed as a revolution in biotechnology, and then derided as a pipe dream. In parallel, investors' enthusiasm for antisense developers has waxed and waned. Along the way, many of the companies that caught the crest of the antisense wave during the early 1990s have vanished or switched to entirely different strategies, but a few have soldiered on. However, the science of antisense has made rather more steady progress toward a better understanding of how gene expression can be regulated artificially. Now, pharmaceutical companies that previously shunned the field are giving it serious consideration, viewing antisense as a potential route for developing novel drugs. The surviving antisense companies are now poised to reap the benefits. Nevertheless, experts caution against a return to the wild exuberance that characterized the early days of antisense.

### Simple theory, complex reality

Antisense inhibition of gene expression relies primarily on the simple rules of base pairing of nucleic acids (see Fig. 1). When a gene is transcribed, the resulting messenger RNA (mRNA) contains the "sense" sequence, which is translated into a protein. Knowing the sequence, it is easy to deduce the complementary, or "antisense", sequence of DNA or RNA that could theoretically bind to a "sense" sequence. Traditionally, antisense sequences have been thought to block the translation of mRNA in one of two ways: either by physically blocking the RNA sequences that the protein translation machinery needs to access, or by marking the RNA molecule for degradation by the enzyme RNase H. RNase H digests the RNA strand of DNA-RNA hybrid molecules, structures that often arise when viruses infect cells. Indeed, naturally, antisense is involved not only in defense against viral attack, but in some types of gene silencing. Targeting the expression of a gene at the RNA level offers cells an additional level of regulatory control, allowing them to switch off the production of proteins even if the RNA is in abundance.

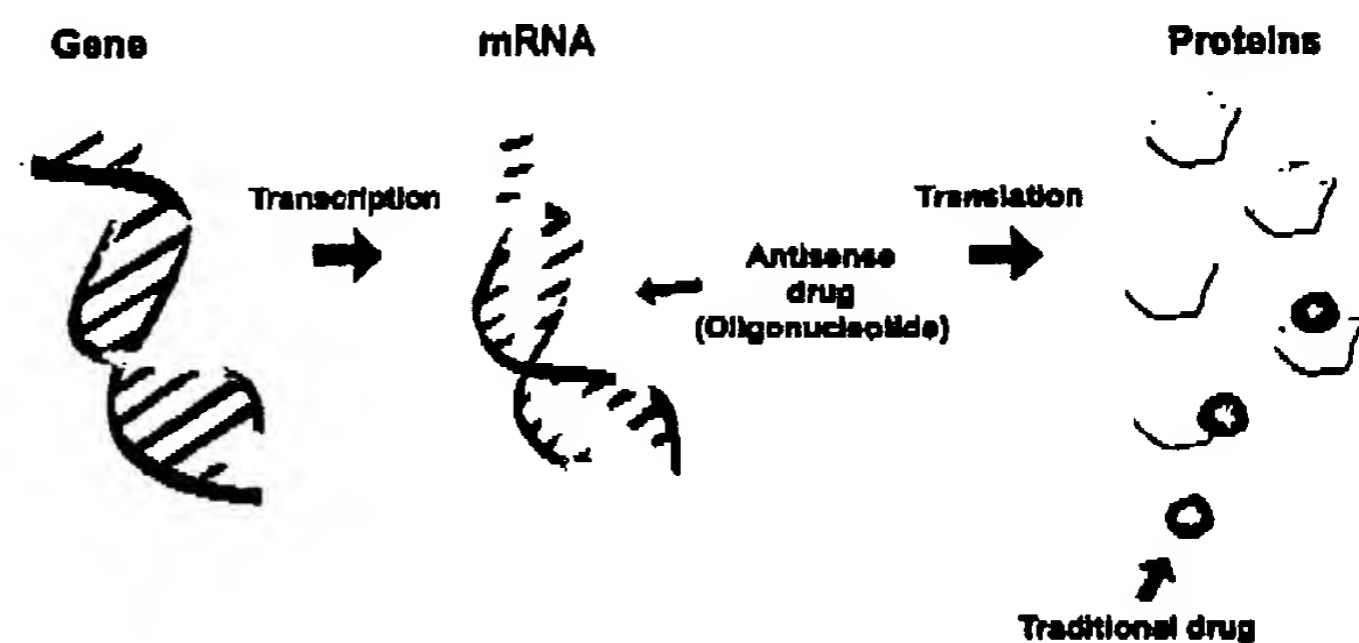


Figure 1. A schematic of the mode of action of antisense drugs. A short oligonucleotide pairs with mRNA that encodes the gene target of interest, preventing its translation into protein. By contrast, traditional small molecule therapeutics block the function of proteins *in situ*. Image concept courtesy of ISIS Pharmaceuticals.

Although not all of the mechanisms underlying antisense inhibition are understood even today, researchers studying the phenomenon in the late 1980s saw the potential for a new type of rational drug design. The idea was to identify genes involved in disease pathogenesis, and then synthesize short oligonucleotides that would shut down the expression of those genes. Because the gene sequence itself would indicate the structure of the (antisense) drug, troublesome drug screening programs would be avoided.

Even though the approach was untested, investors flocked to buy into antisense. "In the early 1990s, there were all these companies, and they all were going to get rich off antisense," says Arthur Krieg, chief scientific officer (CSO) and a founder of Coley Pharmaceuticals (Cambridge, MA). "[But then] the field practically fell into disrepute because of some early results that just weren't reproducible."

Ironically, one of the first problems facing antisense was the fact that it worked too well. The first antisense drugs appeared effective against a whole variety of diseases, ranging from viral infections to tumors. However, more careful controls showed that the drugs' effects were primarily due to nonspecific boosting of the immune system by the oligonucleotides, rather than specific

inhibition of the targeted gene. In other words, antisense appeared to be a single drug with a broad effect, rather than a technology platform capable of producing an entire class of selective drugs.

Krieg and others discovered that a dinucleotide, cytosine-guanine (CG), was responsible for the immune-stimulating actions of oligonucleotides. The dinucleotide is commonly found in viral and bacterial sequences, but is less frequent than expected in human DNA, suggesting that the immune system uses CG as a marker for infection. Although CG dinucleotides are not common genome-wide, many of the successful early antisense drugs were ones that included CG motifs from the human or viral sequences that researchers believed they were targeting.

In addition to the misleading immunostimulatory actions of CG sequences, antisense technology faced other hurdles. Ordinary DNA and RNA oligonucleotides are rapidly degraded by enzymes in the body, and are difficult to deliver to target tissue because, being charged molecules, they are not readily absorbed by many cell types. Even oligonucleotides that have been chemically modified have to be delivered intravenously, limiting their marketability.

As disappointing results mounted, investors fled.

## FEATURE

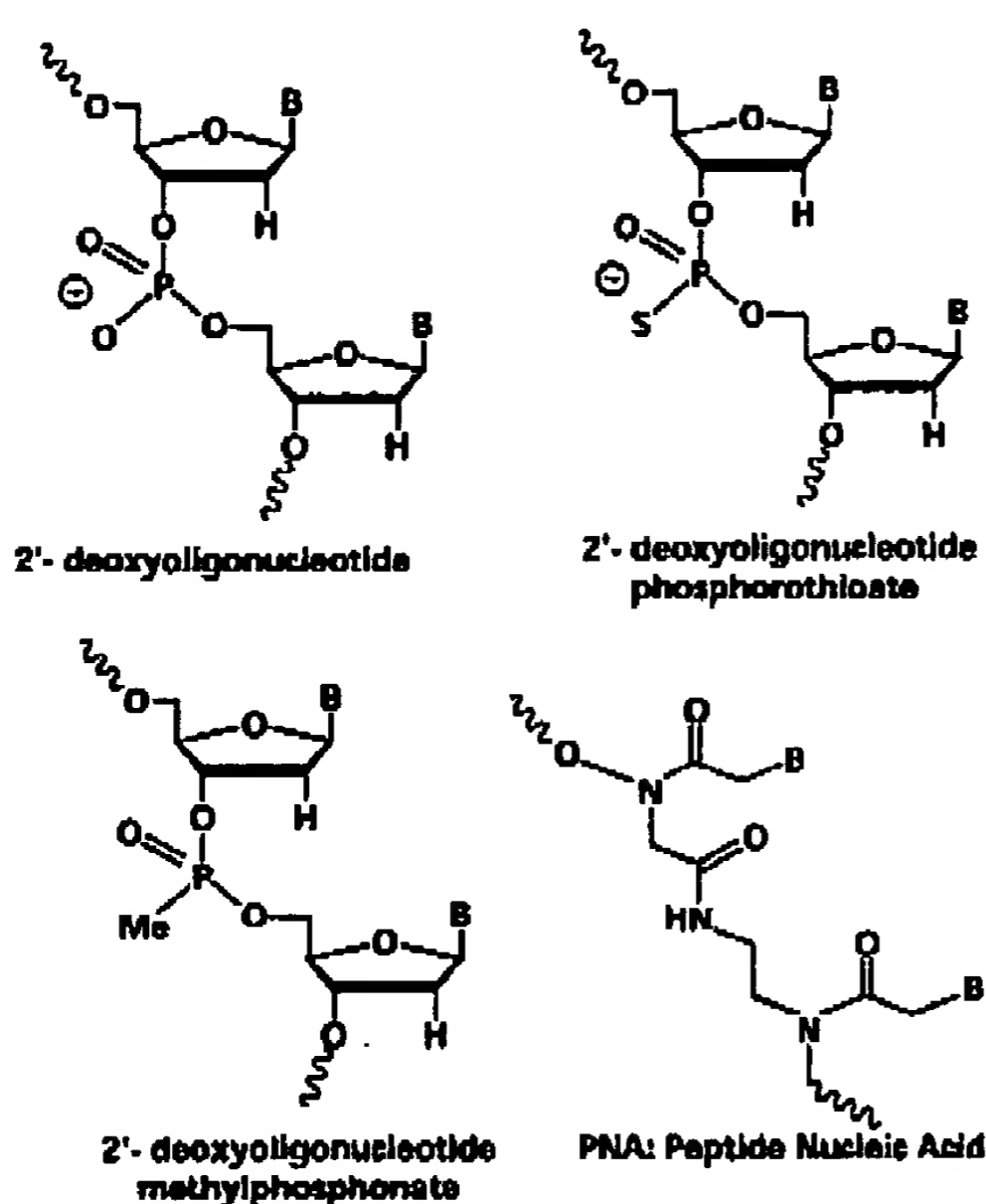


Figure 2. First-generation and second-generation chemistries used to generate antisense oligonucleotides with characteristics more suitable for medicines. Source, *Antisense Drug Technology*, (ed. Crooke, S.T.) p394 (Marcel Dekker, 2001).

One antisense drug, Vitravene—a treatment for cytomegalovirus (CMV)-induced retinitis in AIDS patients—did successfully run the gauntlet of clinical trials, but it remains the only antisense drug on the market. Vitravene meets an important need—albeit a small one—generating about \$157,000 in sales for ISIS Pharmaceuticals (Carlsbad, CA) and Novartis (Basel, Switzerland) last year—a

soon experienced a reversal of fortune when ISIS presented results from its diabetes research program in June 2000. The company presented preclinical data on antisense against two targets: phosphatases p10, a phosphatase that had previously not been implicated in insulin signaling, and phosphatase pdb1b, which had long been of interest to the drug industry. The combination of a new target and an apparently suc-

cessful antisense drug strategy caught the attention of big pharmaceutical companies. Swiftly, Eli Lilly (Indianapolis, IN) signed a \$200 million deal with ISIS in August 2001 (ref. 2). Other large pharmaceutical and biotechnology companies have followed suit, gaining renewed interest and confidence in antisense.

Despite the wild gyrations in investment in the field, the science of antisense has progressed steadily. "I think it was just a continual process of broad advances in the technology coming together in a focused way around a set of targets that people were interested in," says Crooke. "One of the real problems in presenting antisense over the years is that it forced people to ask questions that had never been asked about drugs: How do drugs work? How do you prove mechanism in a real way?"

After the failure of the ISIS 2302 trial, Stanley Crooke, chief executive officer (CEO) of ISIS recalls: "I really wondered for quite a few months whether or not the technology would be delayed in its development by more than a decade."

However, the field

Indeed, as the remaining antisense companies usher a new set of drugs into clinical trials, questions about their mechanism of action continue to nag. "Certainly the principle behind the technology is still true, that you can design an antisense compound for a specific gene target, [but] when that gene has been knocked out, what it does is still an open question," says Sudhir Agrawal, CSO at Hybridon (Cambridge, MA). For example, antisense inhibition of a key regulatory protein could affect a variety of signaling pathways, including some that have yet to be discovered.

Furthermore, even the assumption that a target can be inhibited specifically by an antisense drug is difficult to test. In the early days of the technology, companies assumed that any beneficial effects of experimental antisense drugs were caused by antisense inhibition, something that the field has been "heavily criticized" for.

### A sense of optimism

Furthermore, even the assumption that a target can be inhibited specifically by an antisense drug is difficult to test. In the early days of the technology, companies assumed that any beneficial effects of experimental antisense drugs were caused by antisense inhibition, something that the field has been "heavily criticized" for.

Table 1. Companies pursuing antisense technology

Company (location)	Major antisense focus	Lead product(s) and status
AVI Biopharm (Portland, OR)	Cancer, restenosis	Resten-NG restenosis therapy (c-myc antisense), phase 2
Coley Pharmaceuticals (Cambridge, MA)	CG-based molecules as immune stimulants	Various CG molecules for treating cancer, asthma/allergy, and infectious disease, phase 1 and phase 2
Enzo Biochem (Farmingdale, NY)	Antiviral drugs, agricultural antisense applications	HGT43 (HIV antisense), phase 2
EpiGenesix (Cranbury, NJ)	Respiratory disease	EPI2010 (adenosine A1 receptor antisense), phase 2
Exagenics (Dallas, TX; formerly Cytoclonal)	Target discovery, cancer and infectious disease	OASIS drug screening platform
Genta (Berkeley Heights, NJ)	Various cancers	Genasense (Bcl 2 antisense), phase 3
Hybridon (Cambridge, MA)	Cancer	Gem231 (protein kinase A antisense), phase 2
ISIS Pharmaceuticals (Carlsbad, CA)	Drugs for assorted indications, target discovery Vitravene (CMV retinitis therapy) on market; ongoing target screening collaborations with several partners	
Lorus Therapeutics (Markham, ON, Canada)	Cancer	GT12040 (ribonucleotide reductase antisense), phase 2
Origenix (Laval, PQ, Canada)	Antiviral drugs	ORI1001 (HPV E1 antisense, topical), phase 1
Sequitur (Natick, MA)	Research tools	Various antisense products for functional genomics and drug discovery research, on the market

## FEATURE

according to Frank Bennett, vice president of antisense research at ISIS. Bennett adds: "We always look for a drug's effect on a gene of interest, rather than screening for a phenotypic effect, and assuming it's due to the antisense mechanism."

Researchers have found that one key to getting specific inhibition is to target the correct portion of the target RNA sequence, something that appears to be determined by trial and error only. RNA folds back on itself to form "hairpins" and other complex structures, and not all sites in a given RNA molecule are equally accessible to an antisense inhibitor. Mathematical models that attempt to predict the accessible sites have had limited success. In addition to testing for specific gene expression inhibition, companies are also careful to exclude or modify CG sequences in their oligonucleotides to avoid misleading immunostimulatory effects. However, Cy Stein, associate professor of medicine at Columbia University (New York), remains skeptical about the purported specificity of antisense: "There are certainly non-CG generic mechanisms that are intrinsic to any oligonucleotide [longer than a few bases]."

Although it is possible to identify specific effects of antisense drugs by including sufficient experimental controls, even the more sophisticated new-generation antisense drugs could be working through a variety of mechanisms. "It's sort of like what the Air Force tells you about smart bombs," says Stein. "They're smart, but only up to a

point. But if they're big enough, what do you care?" Properly designed clinical trials can determine whether the drugs work against specific diseases, making quibbling over mechanisms less of a concern.

While some companies try to eliminate the immune stimulation caused by early antisense drugs, Coley Pharmaceuticals hopes to capitalize on it. "If you're trying to develop an antisense [drug] to treat an inflammatory disease ... then the immune activation is not a desirable thing. On the other hand, if you are developing antisense as a way to treat cancer ... it's possible that the immunostimulatory effects might be a desirable effect of the oligo," says Coley's Krieg. The company is now focusing on developing CG-containing oligonucleotides to modulate the immune response in asthma.

Some antisense pioneers also discovered a sideline that could pay the rent during the lean years. "Genomics is producing all these great potential targets, how do you decide what the targets do? Antisense is perfect for that," says Krieg. ISIS's proprietary Genetrove drug-target screening program has blossomed in recent years, and the company is now carrying out screening programs for most of the major pharmaceutical companies. Through Genetrove, drug companies can hire ISIS to do functional analysis of target genes using antisense. "The Genetrove partners get information and they get to use it nonexclusively. We keep the antisense drug rights," says Crooke.

Although the company started the Genetrove program primarily to provide a steady revenue stream, Crooke says that it has also helped to revise hostile attitudes about antisense therapies: "Once you accept that antisense works in cells and animals, and that you might think about a second-generation antisense drug for something like type 2 diabetes, then you're driven to ask 'If it works for this, can it work for other things?'"

### Evolving a backbone

In addition to success in drug screening programs, another factor driving interest in antisense is the development of increasingly stable molecules. DNA and RNA have poor pharmacokinetic profiles as potential drugs as they are swiftly degraded in cells, so researchers in the early 1990s began searching for more durable backbones on which to build antisense molecules. Genta (Berkeley Heights, NJ) initially developed a backbone chemistry called methylphosphonate, although this failed to work in the clinic (see Fig. 2). The first workable antisense DNA backbones—the phosphorothioates—are now the basis for most antisense drugs in clinical trials (see Table 1).

The phosphorothioate or "first-generation" antisense drugs are relatively stable in the bloodstream, and can reach many types of cells when given intravenously. However, according to Frank Bennett at ISIS, "The duration of action of these compounds is not as long as we'd like." To this end, the company has developed a second-generation backbone called the methoxyethyl modification, which increases both the potency and half-life of the drug 5- to 10-fold. At Hybridon, second-generation compounds are made with a backbone that is a mixture of phosphorothioate DNA and RNA. Both ISIS and Hybridon have found a major additional benefit of their second-generation chemistries: "With these types of modifications, we have been able to show that the compounds can be given by oral administration," says Hybridon's Agrawal. Although most experimental antisense drugs are administered intravenously during clinical trials, their oral availability may give antisense a major advantage over other biotechnology-generated therapies.

If there is a third generation of antisense backbones, candidates may include peptide nucleic acids (PNAs, see Fig. 2), which use amino acids as backbone building blocks. PNA proponents argue that peptides can be more easily modified than phosphorothioates, but even researchers developing the technology are cautious in their optimism. "We are still exploring PNA applications internally. It's been more difficult to

### Interfering RNA

Discovered in 1998, the phenomenon known as RNA interference (RNAi) has rapidly become a standard technique for many biologists. "It's been pretty revolutionary. It's almost like PCR," says Brenda Bass, a researcher in the Howard Hughes Medical Institute at the University of Utah (Salt Lake City, UT). RNAi involves introducing a double-stranded piece of RNA matching a target gene's sequence into a cell. By poorly understood mechanisms, the cell then degrades messenger RNA containing that sequence, rapidly and specifically shutting down expression of the gene.

RNAi appears to act by a different mechanism than antisense, and its effects are more specific and thorough, but until recently there was a major catch: RNAi did not work in mammalian cells. While researchers working on fruitflies or worms enjoyed a treasure trove of new functional information uncovered by RNAi "knockouts", those working in mammalian systems could only watch in amazement.

But in May last year, researchers at the Max Planck Institute (Göttingen, Germany) reported that small RNA molecules, between 21 and 23 base pairs in length, which they dubbed siRNAs, could shut down the expression of specific genes in mammalian cells like RNAi does in worms<sup>4</sup>. "So far it looks like it's more reproducible than antisense," says Bass, but she cautions that the technique is still very new.

Companies are also enthusiastically rushing into the breach. AGY Therapeutics (S. San Francisco, CA) announced on November 15, 2001 that its researchers had successfully blocked neuronal gene expression in mammalian cells, and the company plans to use the technology to develop therapies for CNS diseases. Given the history of the antisense field, though (see main text), caution may be appropriate. "It's just as likely [to work therapeutically] as antisense," says Bass, "and then you can decide if you think that's likely." AD



## FEATURE

## The sense of intellectual property

Sometimes, it pays to believe in a lost cause. That is the lesson that ISIS learned as one of the early entrants in the antisense field. When antisense became a dirty word on Wall Street, ISIS took advantage of rock-bottom prices to purchase patent rights from other companies, locking up what appears to be the dominant intellectual property portfolio in the field.

"It's hard to believe that an opportunity of this scale would have been dominated by one little company this way. Today, I think we own most of what's relevant to own," says Stanley Crooke, CEO of ISIS Pharmaceuticals (Carlsbad, CA).

Crooke's boast is not an idle one. Although ISIS developed several important antisense technologies, it also aggressively bought the rights to other companies' patents, including the patent estate of former antisense company Gilead (Foster City, CA), and rights to key patents from Hybridon (Cambridge, MA). "Our goal was to create a thicket of patent claims that would make it cheaper to stop and shop at ISIS than to try to drive around our patent estate," says Crooke. A company could still produce "first-generation" antisense drugs using technology that is in the public domain (see main text), but more advanced chemistries are owned or licensed by ISIS.

On the other hand, developing an antisense drug requires more than just a backbone. "The scientist or company who owns a gene [patent] owns that antisense as well," explains Sudhir Agrawal, CSO at Hybridon. The owner of the gene patent and the owner of the antisense backbone patent would have to cooperate in order to develop the drug, explaining the eagerness of antisense companies to pursue studies of gene function and identify new potential drug targets.

Whether this situation will drive companies toward collaboration or litigation remains to be seen. AD

advance than second-generation chemistry [and] the pharmacokinetics for PNAs aren't as attractive as they are for second-generation chemistry," says Bennett. ISIS owns an exclusive license for therapeutic applications of PNAs, but has sublicensed the right to develop PNA-based antisense antibiotics to Pantheoco (Copenhagen, Denmark). Pantheoco hopes to begin clinical trials on the first PNA antibiotics this year.

Even if newer backbone chemistries do not find applications in the clinic, they may be useful in the laboratory. Efforts to use antisense for studies of gene function have already led to some provocative breakthroughs, including the development of inhibitory RNA (RNAi) techniques in a wide range of model systems (see, "Interfering RNA").

## The boundaries of sense

As two generations of antisense drugs move through clinical trials, investors and researchers are being reminded of the reasons the technology looked good in the first place: low toxicity, low production costs (using traditional oligonucleotide synthesis techniques), and potentially easier drug discovery, because simply knowing the target gene sequence leads directly to the drug. At the same time, ongoing research has defined the limits of antisense with even greater clarity.

At very high doses, antisense drugs activate the immune system's complement cascade, resulting in potentially serious side effects. However, the more potent drugs now in company pipelines are effective at

doses far lower than those capable of triggering such side effects. There may be other concerns surrounding the intravenous administration of antisense drugs, such as an increased risk of infections in the delivery lines. Stein hypothesizes that this effect, which has been seen in some clinical trials, could be caused by negatively charged oligonucleotides causing the aggregation of white blood cells near the site of injection, but adds that it "seems to be manageable."

Other barriers are not as easily surmounted. For example, "it would be nice to be able to target some of the technology towards cardiac tissues," says Bennett, but oligonucleotides do not appear to reach skeletal or cardiac muscles from the bloodstream. ISIS is now characterizing the pathways by which oligonucleotides penetrate cells, although Bennett says that it is more of a descriptive science at this stage. Antisense compounds currently in development are unable to penetrate the blood-brain barrier, making it unlikely that they will be used to treat diseases of the central nervous system (CNS). There is, however, preliminary evidence that PNA-based antisense drugs might be able to reach the CNS (ref. 3).

Antisense will also not be useful for treating acute conditions, in which a protein must be eliminated rapidly. If an antisense drug is indeed working by an antisense mechanism, the actions of the gene product will not be terminated until the existing protein pool has been degraded by the cell—a process that could take hours or even days.

However, antisense has delivered some pleasant surprises, including the finding that oligonucleotides can be delivered easily through the skin and by inhalation into the lungs. "When we began the company we never dreamt that we could have an antisense that we could just rub on the skin and deliver into the skin structures," says Bennett, referring to promising results from animal tests on the company's CD80/CD86-inhibiting antisense treatment for psoriasis.

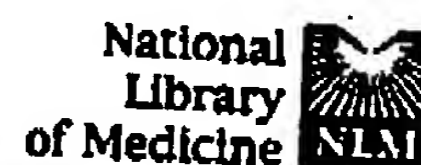
## Sense from nonsense

In the next few years, experts in the field expect antisense to continue to make steady progress, and there is little doubt that more antisense drugs will soon join Vitravene on the market. Even ISIS 2302, the drug whose failure nearly wiped out ISIS in early 2000, is now in a restructured phase 3 trial for the treatment of Crohn's disease. Crooke says that the first trial failed because of a dosing problem that had not previously been identified, and is optimistic that the drug will now succeed.

On the corporate side, ISIS, Hybridon, and Genta are usually identified as the survivors in the antisense field, remaining most heavily committed to investing in antisense. Among the three companies, ISIS appears to have locked up much of the intellectual property in the field (see, "The sense of intellectual property"). However, ISIS will still have their competitors: antisense drugs are, after all, just another type of medicine. On November 19, 2001, ISIS announced that it had initiated a phase 2 clinical trial on ISIS 104838, the first second-generation antisense drug to enter the clinic. The drug, an inhibitor of tumor necrosis factor  $\alpha$ , is being tested intravenously against rheumatoid arthritis. However, ISIS 2302 will compete directly with the Centocor's blockbuster drug Remicad in the Crohn's disease market. Other antisense cancer therapies, such as Genta's Genasense (in phase 3 trials against several cancers), are being studied primarily as chemosensitizers, to enhance the effects of chemotherapy agents.

Still, even skeptics who have watched the field develop have been amazed by its progress so far. "In my wildest dreams I didn't think we were going to get this far. I admit to what I like to call a healthy skepticism about [antisense] succeeding clinically, but when you look at it, why not?" says Stein.

1. Dove, A., *Nat. Biotechnol.* 18, 19 (2000).
2. Miller, E., *Nat. Biotechnol.* 18, 888-889 (2001).
3. Tyler, B.M. et al., *Proc. Natl. Acad. Sci. USA* 98, 7063-7068 (1999).
4. Sayda, M.E. et al., *Nature* 411, 494-496 (2001).



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1: In Vivo 1995 Sep-Oct;9(5):469-74

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# **Expression of decorin mRNA in the skin of patients with ossification of the posterior longitudinal ligament.**

Ishidou Y, Tokunaga M, Murata F, Yoshida H, Sakou T.

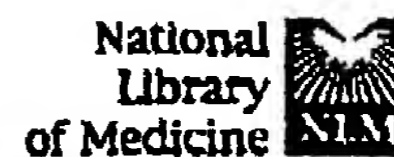
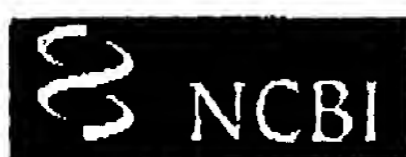
Department of Orthopaedic Surgery, Kagoshima University, Japan.

Ossification of the posterior longitudinal ligament (OPLL) is an intractable disease developing into severe myelopathy and often accompanied by ossification of several other spinal ligaments and articular ligaments. Therefore, it is highly probable that predisposition to systemic ossification underlies the onset of this disease. Terayama et al(1981) reported that the nuchal skin of OPLL patients tended to be tougher than that of healthy individuals. Decorin is a component of the extracellular matrix which antagonistically regulates the action of Transforming growth factor-beta (TGF-beta). Inamura et al(1995) demonstrated immunohistochemically that decorin increased in the epidermis of the nuchal skin of OPLL patients. This suggests an abnormal expression of the extracellular matrix in the skin of OPLL patients. In the present study, in situ hybridization with non-radioactive synthetic oligodeoxynucleotide probes revealed an enhanced expression of decorin mRNA in the epidermal keratinocytes of OPLL patients. The increased expression of decorin mRNA in the epidermis of OPLL patients may be interpreted as reflecting abnormalities of the matrix associated with ossification of the ligaments. Studies on the role of decorin in ossification will contribute to clarification of the pathophysiology and pathogenesis of OPLL.

PMID: 8900925 [PubMed - indexed for MEDLINE]

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### Detection of rabbit haemorrhagic disease virus (RHDV) by in situ hybridisation with a digoxigenin labelled RNA probe.

Gelmetti D, Grieco V, Rossi C, Capucci L, Lavazza A.

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy.

An in-situ hybridisation (ISH) technique for the detection of rabbit haemorrhagic disease virus (RHDV) was developed. Thirteen seronegative adult rabbits were infected oro-nasally using the BS89 RHDV strain. Liver and spleen samples were collected from 4 h post infection (p.i.) and repeated every 4 h till 44 h p.i. Each sample was tested immunohistochemically, by sandwich ELISA and by ISH. A 2.482-kb RNA probe, matching the genomic fragment coding for the VP60 structural protein of RHDV, was arranged. Two RNA probes (sense and antisense) were transcribed in vitro and UTP-digoxigenin-labelled. The antisense probe clearly detected positivity in the cytoplasm of the hepatocytes at 8 h p.i. Labelled hepatocytes were scattered throughout the sections until 24 h p.i. followed by a more diffuse perilobular positive reaction. A much weaker signal of similar distribution was detected up to 24 h p.i. using the sense RNA probe. All spleen samples tested negative for both probes. Liver samples were positive at 32 h p.i. using both ELISA and the immunoperoxidase test. Spleen samples were positive using only the ELISA at 32 h p.i. This study showed that RHDV replication occurred almost immediately after inoculation and that the liver appears to be the main site of replication.

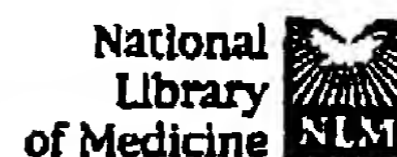
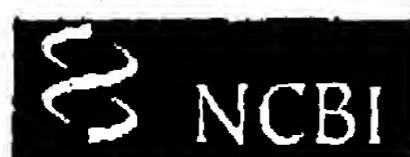
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1: Anticancer Res 1995 May-Jun;15(3):761-7

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### Detection of O6-methylguanine-DNA methyltransferase-expression in human tumors by means of in situ hybridization.

Eberhagen I, Bankmann M, Kaina B.

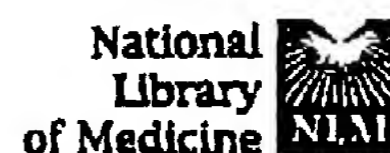
Research Center Karlsruhe, Department of Genetics, Karlsruhe, Germany.

The DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) is a main determinant of resistance of tumor cells towards nitrosoureas that are in use as cytostatic drugs. It therefore appears of importance to determine its expression in tumors before the utilization of nitrosoureas for tumor treatment. We have determined the level of expression of MGMT in mammary carcinomas and brain tumors by means of in situ hybridization, using digoxigenin-labeled RNA driven by MGMT expression vector in sense and antisense orientation. It is shown that the intensity of the hybridization signal correlates with the MGMT activity of a given tumor. The results demonstrate the applicability of in situ hybridization for determination of MGMT expression in tumor sections.

PMID: 7645955 [PubMed - indexed for MEDLINE]

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**Detection and quantification of Epstein-Barr virus EBER1 in EBV-infected cells by fluorescent in situ hybridization and flow cytometry.**

**Stowe RP, Cubbage ML, Sams CF, Pierson DL, Barrett AD.**

Department of Pathology, University of Texas Medical Branch, Galveston 77555-0605, USA.

A rapid and highly sensitive fluorescent in situ hybridization (FISH) assay was developed to detect Epstein Barr virus (EBV)-infected cells in peripheral blood. Multiple fluorescein-labeled antisense oligonucleotide probes were designed to hybridize to the EBER1 transcript, which is highly expressed in latently infected cells. After a rapid (30 min) hybridization, the cells were analyzed by flow cytometry. EBER1 was detected in several positive control cell lines that have variable numbers of EBV genome copies. No EBER1 was detected in two known EBV-negative cell lines. Northern blot analyses confirmed the presence and quantity of EBER1 transcripts in each cell line. This method was used to quantify the number of EBV-infected cells in peripheral blood from a patient with chronic mononucleosis. These results indicate that EBV-infected cells can be detected at the single cell level, and that this assay can be used to quantify the number of EBV-infected cells in clinical samples.

PMID: 9820577 [PubMed - indexed for MEDLINE]

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